Induction of a Reversible Cardiac Lipidosis by a Dietary Long-Chain Fatty Acid (Erucic Acid)

Relationship to Lipid Accumulation in Border Zones of Myocardial Infarcts

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Previous studies have demonstrated that cardiac myocytes in the border zone of acute myocardial infarction become markedly overloaded with neutral lipid during the transition from reversible to irreversible injury. To examine directly the role of these changes in neutral lipid metabolism in the development of irreversible cellular injury and associated increases in tissue Ca^{2+} content, the authors fed rats large amounts of a fatty acid (erucic acid) that is poorly oxidized by the heart and that subsequently accumulates as neutral lipid. Rats fed a high erucic acid (C22:1) diet in the form of 20% rapeseed oil for 3–5 days had a fourfold increase in triglyceride (49.5 ± 3.8 SEM mg/g wet wt versus 13.6 ± 13, n = 4) and a 60% increase in long-chain acyl CoA content (166.0 ± 21.9 versus 91.5 ± 9.0 nM/g wet wt, n = 4), compared with controls. However, there was no change in long-chain acyl carnitine or total phospholipid content. Histochemical studies showed accumulation of numerous lipid droplets in the myocytes, and electron microscopy revealed localization of lipid vesicles in direct contact with mitochondria, thus mimicking the lipid-laden cells in the border zone regions of acute myocardial infarcts. The acute lipidosis was reversible with either continued feeding of erucic acid for several weeks or conversion to a normal diet. It was not associated with an increased tissue Ca^{2+} content, nor with cell necrosis. However, continued erucic acid intake for 3 months was associated with focal myocardial degeneration and loss of myocytes. These results suggest that acute increases in neutral lipids, as found in the border zone of acute myocardial infarction, may not be the cause of progression to irreversible damage during acute myocardial injury, but that the persistent presence of similar lipid material over months may result in focal myocardial degeneration. (Am J Pathol 1983, 112:68–77)

RECENT STUDIES have suggested that alterations in both neutral lipid and phospholipid metabolism can contribute to the development of irreversible injury in ischemic myocardium.[1-12] In ischemic canine myocardium, there is a close correlation between degradation of membrane phospholipids, the presence of an *in vitro* sarcolemmal Ca^{2+} permeability defect, and the uptake of technetium-99m pyrophosphate.[2] Hydrolysis of membrane phospholipids by exogenous or endogenous phospholipases is capable of producing a marked increase in Ca^{2+} permeability in membrane vesicles isolated from control tissue.[13] Reduction of ischemia-induced membrane permeability is associated with improved ultrastructure and function in isolated perfused myocardium.[14] A similar permeability defect in liver can be prevented *in vivo* by limiting the degradation of membrane phospholipids and can be reversed by reconstitution of the membrane with exogenous phospholipids.[1]

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In addition to these changes in phospholipid metabolism, myocardial ischemia results in marked increases in other lipid metabolites that may be involved in the production of irreversible cell injury.[4-11] As a consequence of the inhibition of fatty acid oxidation, there is a substantial increase in long-chain fatty acyl CoA and carnitine derivatives, both of which have potent detergent properties.[3-8] Previous studies have demonstrated a close correlation between the accumulation of long-chain fatty acyl CoA and the development of mitochondrial dysfunction.[6] In addition, several studies have suggested that the high levels of free fatty acids and other neutral lipids may directly induce decreased myocardial function during ischemia.[15-22] Pharmacologic intervention with agents designed to decrease the accumulation of free fatty acid moieties have been demonstrated to have beneficial effects in several different models of myocardial ischemia.[23,24] The accumulation of unoxidized neutral lipids has characterized several types of muscle cell injury.[3,22-27] In particular, experimental studies of the border zone of acute myocardial infarction have demonstrated the massive intracellular accumulation of neutral lipid droplets. Lipid droplets were identified in cells with features of irreversible injury as well as in cells with less severe ultrastructural changes.[27]

The purpose of the present study was to evaluate the role of marked increases in triglyceride and long-chain acyl CoA content on the development of irreversible myocardial injury and the associated increases in tissue Ca^{2+} content that frequently accompany this process. Previous investigations have demonstrated that the feeding of erucic acid as 20% rapeseed oil induced a marked cardiac lipidosis in rat myocardium.[28-33] Erucic acid is a long-chain fatty acid with one unsaturated carbon-carbon bond (C22:1). This fatty acid has been demonstrated to be oxidized poorly by rat myocardium.[34-38] Because the changes in lipid metabolism are predominantly confined to neutral lipids, this model offers the opportunity to assess their ability to produce irreversible injury independent of changes in membrane phospholipids.

Materials and Methods

Protocol

Male Sprague-Dawley rats (100-150 g) were fasted overnight and then placed on one of two diets (obtained from Nutritional Biochemical Co.): 1) a control diet, consisting of rodent chow with 20% corn oil or 2) the test diet, consisting of rodent chow with 20% rapeseed oil. After various periods on the rapeseed oil diet, some rats were switched to the control diet. The animals were killed by cervical dislocation and the hearts quickly isolated for various biochemical measurements and morphologic examination. At various time points, two to four hearts were obtained for biochemical analyses, and four hearts were obtained for morphologic analysis and calcium measurements.

Lipid Analyses

The preparation of the tissue for acyl CoA and carnitine analyses was similar to that previously reported by Shug et al.[7] Hearts were quickly isolated and frozen immediately between aluminum blocks precooled in liquid nitrogen (N_2). The tissue was weighed quickly and powdered in a percussion stainless steel mortar precooled in liquid N_2. The samples were homogenized immediately in 5 volumes of ice-cold 6% perchloric acid and spun at 25,000g in a Beckman J-21B centrifuge for 20 minutes. The pellet was washed twice with 6% perchloric acid and once with distilled water and suspended in 7 ml distilled water containing β-mercaptoethanol. Saponification of the acyl derivatives in the supernatant and pellet was carried out at alkaline pH (11.0-12.0) by incubation at 70 C for 2 hours for carnitine analyses and 55 C for 15 minutes for CoA analyses. After neutralization, the extracts were measured for carnitine by the method of McGarry and Foster[39] and for CoA by a NADH-linked citrate cleavage enzyme system.[40] As assessed by the addition of internal standards, the percentage of recovery of all CoA and carnitine derivatives was in excess of 75%.

For the phospholipid and triglyceride analyses, frozen heart tissue was minced in 5 ml 0.1 M Tris, 0.1 M KCl, 0.5 mM EDTA buffer, pH 7.4. Aliquots of the homogenate (500 μl) were extracted for lipid analysis by the method of Bligh and Dyer.[41] Lipid extracts were concentrated under N_2 gas at 60 C, and total phospholipid content was measured as previously described.[1] Lipid phosphate (PO_4) was measured by the method of Rouser.[42] For isolation of individual phospholipid species, the dried lipid was resuspended in 100 μl of chloroform:methanol (1:1), and 50-μl aliquots were spotted on 0.43 mM silica gel G-precoated plates (ANALTECH) and chromatographed in a solvent mixture of chloroform, methanol, acetic acid, and water (65:25:2:4). The lipids were visualized in I_2 vapor. The spots were scraped from the plates and used directly for PO_4 determination without prior elution. Triglycerides were measured in the lipid extracts by the previously described method of Fletcher.[43]

Calcium Measurements

Fresh tissue samples were dried overnight in a 105 C oven. The dried samples were weighed and wet-washed in a solution of 0.1 N nitric acid. Aliquots of the samples and standard solutions were diluted in distilled water containing 1% lanthanum chloride. Calcium content was measured by atomic absorption spectrophotometry.[44]

Morphology and Histochemistry

Transverse sections of the ventricles were fixed in 10% formalin in 0.1 M phosphate buffer. Some blocks were embedded in paraffin, and 6-μ sections were cut from these blocks and stained with hematoxylin and eosin. Other blocks were embedded in methacrylate, and 2-μ sections were cut and stained with Lee's stain.[45] Additional blocks were frozen, and 6-μm sections were cut in a cryostat and stained for lipids with oil red O.[46] Photomicrographs were prepared with a Zeiss Photomicroscope III.

Small blocks were immersed in 3% glutaraldehyde in 0.1 M phosphate buffer, washed in phosphate buffer, postfixed in 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in acetone, and embedded in Epon-Araldite.[47] Sections were cut at 1.5 μ and stained with toluidine blue. Thin sections of these blocks were cut, mounted on copper grids, and stained with uranyl acetate and lead citrate. The sections were examined, and electron micrographs were prepared with the use of a JEOL 100S electron microscope.

Statistical Analysis

A two-tailed Student t test was used. The results were considered significant when $P < 0.05$.

Results

Lipid Analyses

In the rats fed a diet containing 20% rapeseed oil (an oil composed of approximately 50% erucic acid) triglyceride rapidly accumulated within the myocardium. As revealed in Table 1, 3–5 days of feeding resulted in a fourfold increase in triglyceride content in the hearts of the erucic-acid-treated rats, compared with hearts of control rats (20% corn oil diet). However, this marked change in neutral lipid content was not accompanied by any detectable change in total phospholipid content (Table 1). Thin-layer chromatography of the total phospholipid extract (Table 2) revealed that there was a slight decrease in phosphatidyl ethanolamine content in the erucic-acid-treated hearts but that slight increases in sphingomyelin and cardiolipin content compensated for this alteration. These changes are in agreement with previous studies.[48,49]

Table 1 – Phospholipid and Triglyceride Content of the Myocardium of Rats Fed the Control and Erucic Acid Diets*

	Triglyceride (mg/g wet wt)	Total phospholipid content (μ/g wet wt)
Control (n = 4)	13.6 ± 1.3 (SEM)	10.8 ± 0.4
Erucic (n = 4)	49.5 ± 3.8†	10.4 ± 0.5‡

* The animals were starved for 24 hours and then fed a 20% corn oil (control) or 20% rapeseed oil (erucic) diet for 3–5 days. The hearts were isolated and extracted for lipids, and triglyceride and phospholipid content was measured as described in Materials and Methods.
† $P < 0.05$ versus control.
‡ Not significant ($P > 0.05$) versus control.

The marked change in triglyceride content in the erucic-acid-treated hearts was not associated with any detectable increases in acid-soluble or acid-insoluble carnitine content (Table 3). There was a 60% increase in long-chain acyl CoA content (acid-insoluble CoA) in the erucic-acid-treated hearts versus corresponding control hearts (Table 3). The total CoA content of the erucic-acid-treated hearts was also elevated.

Tissue Ca^{2+} Content

The acute alterations in neutral lipid metabolism were not associated with a change in tissue Ca^{2+} content. As shown in Figure 1, there was little difference in tissue Ca^{2+} between the erucic-acid-treated and control hearts up to 8 weeks of feeding.

Morphology and Histochemistry

The rats were killed for these studies at 1, 3, 5, 10, 16, 28, 44, 56, 72, and 98 days after starting the different diets. Morphologic lesions and abnormal lipid accumulation were not detected in the hearts of the control rats. In the erucic-acid-treated rats, the cardiac myocytes showed moderate accumulation of oil-red-O-positive lipid droplets after 1 day and marked accumulation of lipid droplets after 3 and 5 days (Figure 2). In paraffin sections stained with hematoxylin and eosin, the cardiac myocytes did not show evidence of necrosis, and no inflammatory infiltrates were present. Electron-microscopic examination revealed numerous cytoplasmic lipid droplets in

Table 2—Phospholipid Composition of the Myocardium of Rats Fed the Erucic Acid and Control Diet*

	Cardiolipin	Phosphatidyl ethanolamine	Phosphatidyl choline	Sphingomyelin	Origin
Control† (n = 4)	1.49 ± 0.23 (14%)	3.53 ± 0.22 (33%)	4.24 ± 0.07 (39%)	0.90 ± 0.09 (8%)	0.55 ± 0.15 6%)
Erucic† (n = 4)	1.76 ± 0.30 (17%)	2.71 ± 0.18 (26%)	4.04 ± 0.41 (39%)	1.25 ± 0.14 (12%)	0.55 ± 0.09 (6%)

* The animals were starved for 24 hours and then fed a 20% corn oil (control) or 20% rapeseed oil (erucic) diet for 3-5 days. The hearts were isolated and extracted for lipids, and triglyceride and phospholipid content was measured as described in Materials and Methods.
† All values are reported in micromoles per gram wet weight ± SEM. All values in the group fed the erucic acid diet are not significant ($P > 0.05$) as compared with the control group.

Table 3—Long-Chain Fatty Acyl Derivatives in the Myocardium of Rats Fed the Control and Erucic Acid Diets*

	Carnitine†		CoA§	
	Acid-soluble	Acid-insoluble	Acid-soluble	Acid-insoluble
Control (n = 4)	1.56 ± 0.08	0.165 ± 0.004	18.2 ± 2.8	91.5 ± 9.0
Erucic (n = 4)	1.54 ± 0.15‡	0.180 ± 0.010‡	29.3 ± 3.4‖	166.0 ± 21.9‖

* The animals were starved for 24 hours and then fed a 20% corn oil (control) or 20% rapeseed oil (erucic) diet for 3-5 days. The hearts were isolated and extracted for lipids, and long-chain fatty acyl derivatives were measured as described in Materials and Methods.
† Micromoles per gram wet weight ± SEM.
‡ Not significant ($P > 0.05$) versus control.
§ Nanomoles per gram wet weight ± SEM.
‖ $P < 0.05$ versus control.

close association with the mitochondria of the cardiac myocytes (Figures 3 and 4). The myocytes showed slight alterations, including slight swelling of the mitochondria and loss of glycogen. The latter alterations were detected in control preparations and were attributed to the sampling procedure and immersion fixation.

In spite of continued feeding of the rapeseed oil diet, the cardiac myocytes showed less severe lipid accumulation at Day 10, and only mild focal lipid accumulation at Days 16 to 56 (Figure 5). At these time points, no lesions were detected in the paraffin- and plastic-embedded sections on light-microscopic examination. Similar observations were made in rats switched from the rapeseed oil diet to the control diet. At Day 72 of continued feeding of the rapeseed oil diet, the myocardium exhibited focal lesions characterized by degeneration and loss of myocytes and accumulation of macrophages (Figure 6A). Focal accumulation of lipid droplets in cardiac myocytes also was present at this time (Figure 6B). After 98 days of erucic acid treatment, the myocardium contained focal and small scars composed of loose fibrous tissue (Figure 7). Foci of myocytes with prominent lipid accumulation were not present.

Discussion

The present study demonstrates that the administration of dietary erucic acid as 20% rapeseed oil results in the marked accumulation of triglycerides in rat myocardium. This result is in agreement with the findings of several previous studies that have documented that erucic acid accumulates in rat myocardium as neutral lipid droplets.[28,50] The basis for this large accumulation of neutral lipid probably is the poor oxidation of erucic acid by heart mitochondria.[34-38] The work of Bremer et al with isolated rat heart mitochondria has demonstrated that erucic acid is readily transported by the mitochondrial acyl carnitine: CoA transferase, but that the fatty acid accumulates within the mitochondrial matrix as

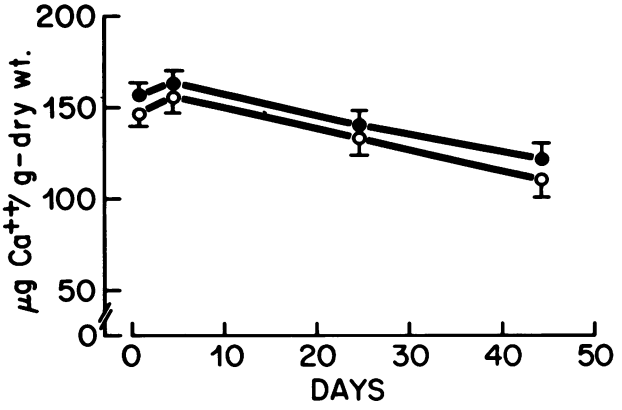

Figure 1—Ca^{2+} content of myocardium from rats fed a control diet (20% corn oil) (*open circles*) and an erucic-acid-enriched diet (*solid circles*). There are no significant differences between the two groups at the time points examined (n = 4 in each group).

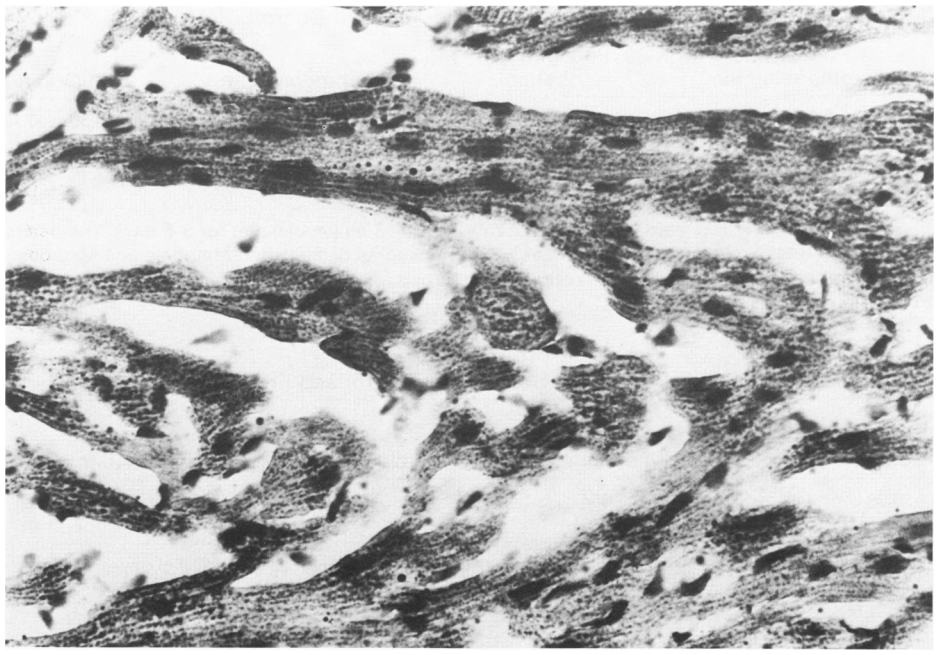

Figure 2—Light-microscopic photomicrograph of myocardium from a rat fed a erucic acid diet for 3 days. The cardiac myocytes contain numerous darkly stained lipid droplets. (Frozen section, oil red O, ×360)

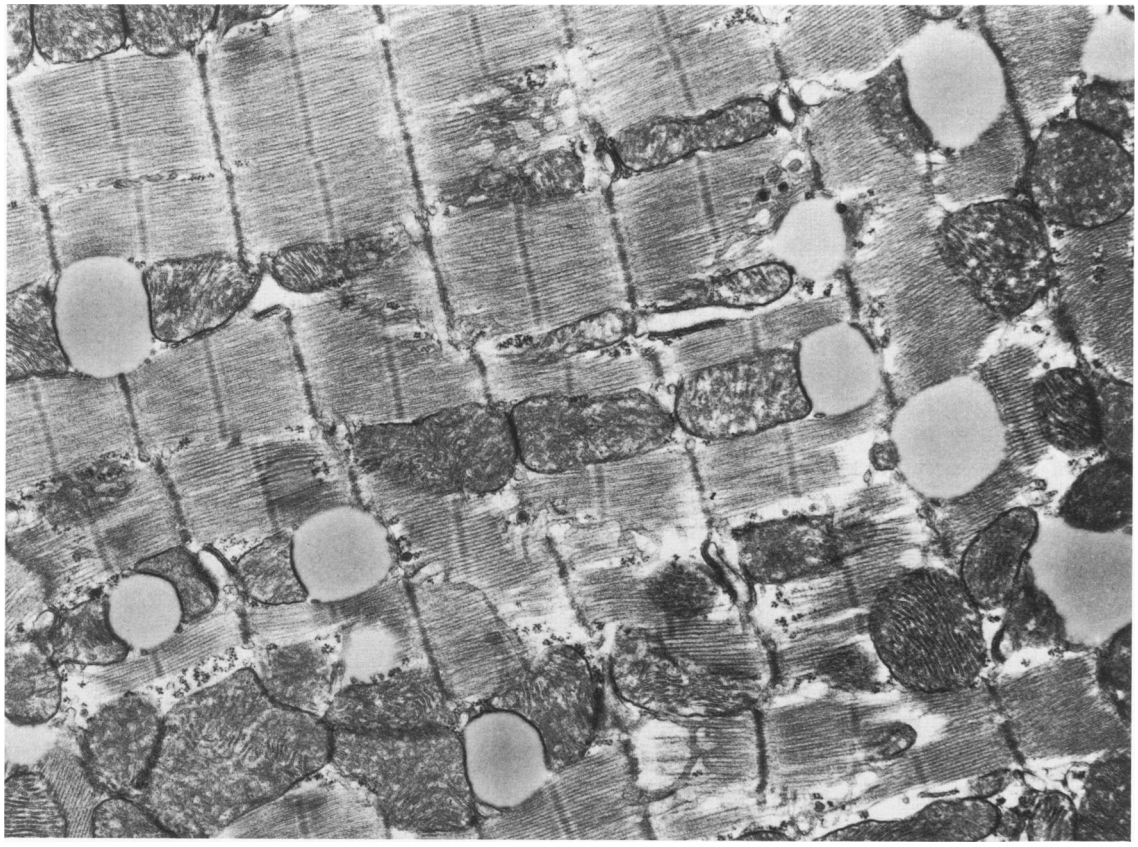

Figure 3—Electron micrograph of a cardiac myocyte from a rat treated with erucic acid for 3 days. Note the numerous lipid droplets and general preservation of ultrastructure. (×15,000)

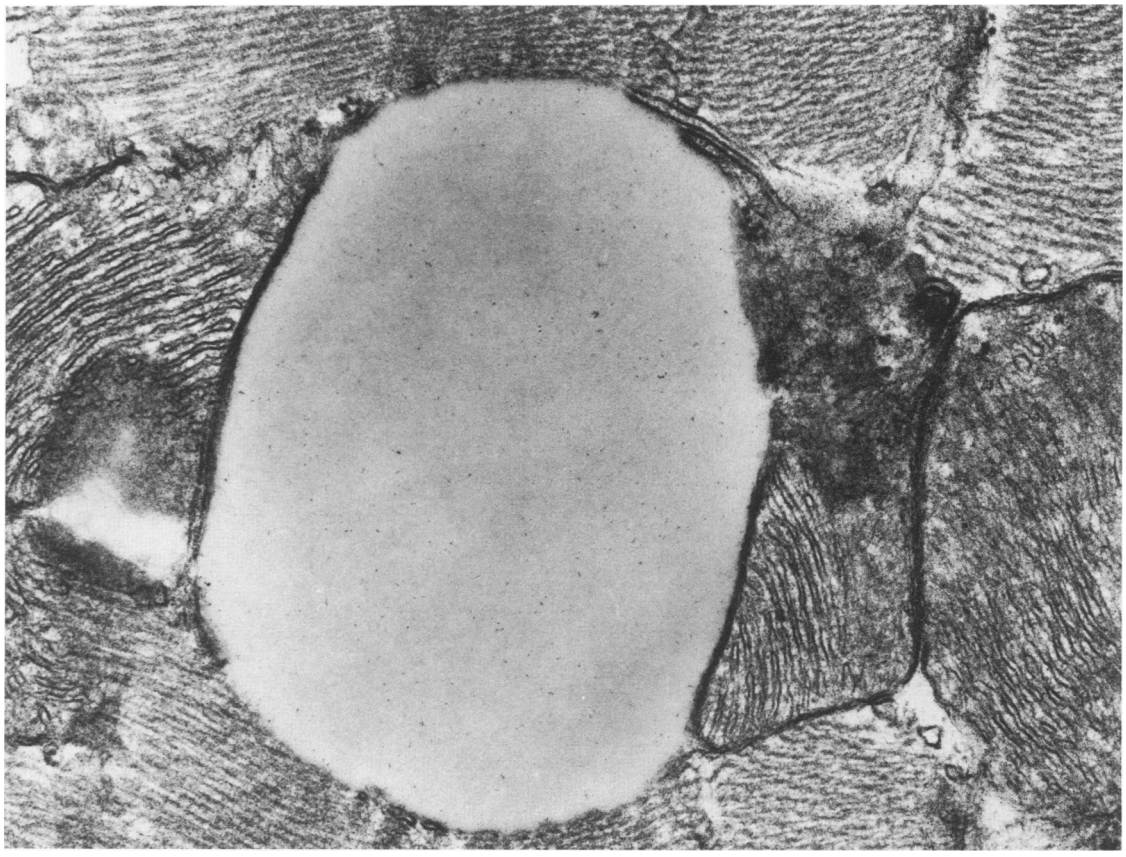

Figure 4 — Electron micrograph of a microregion of a cardiac myocyte from a rat treated with erucic acid for 3 days. Note the close opposition of a typical cytoplasmic lipid droplet and the adjacent mitochondria. (×44,200)

Figure 5 — Light-microscopic photomicrograph of myocardium from a rat fed a erucic acid diet for 28 days. Occasional cells contain a few lipid droplets (*arrows*). (Frozen section, oil red O, ×360)

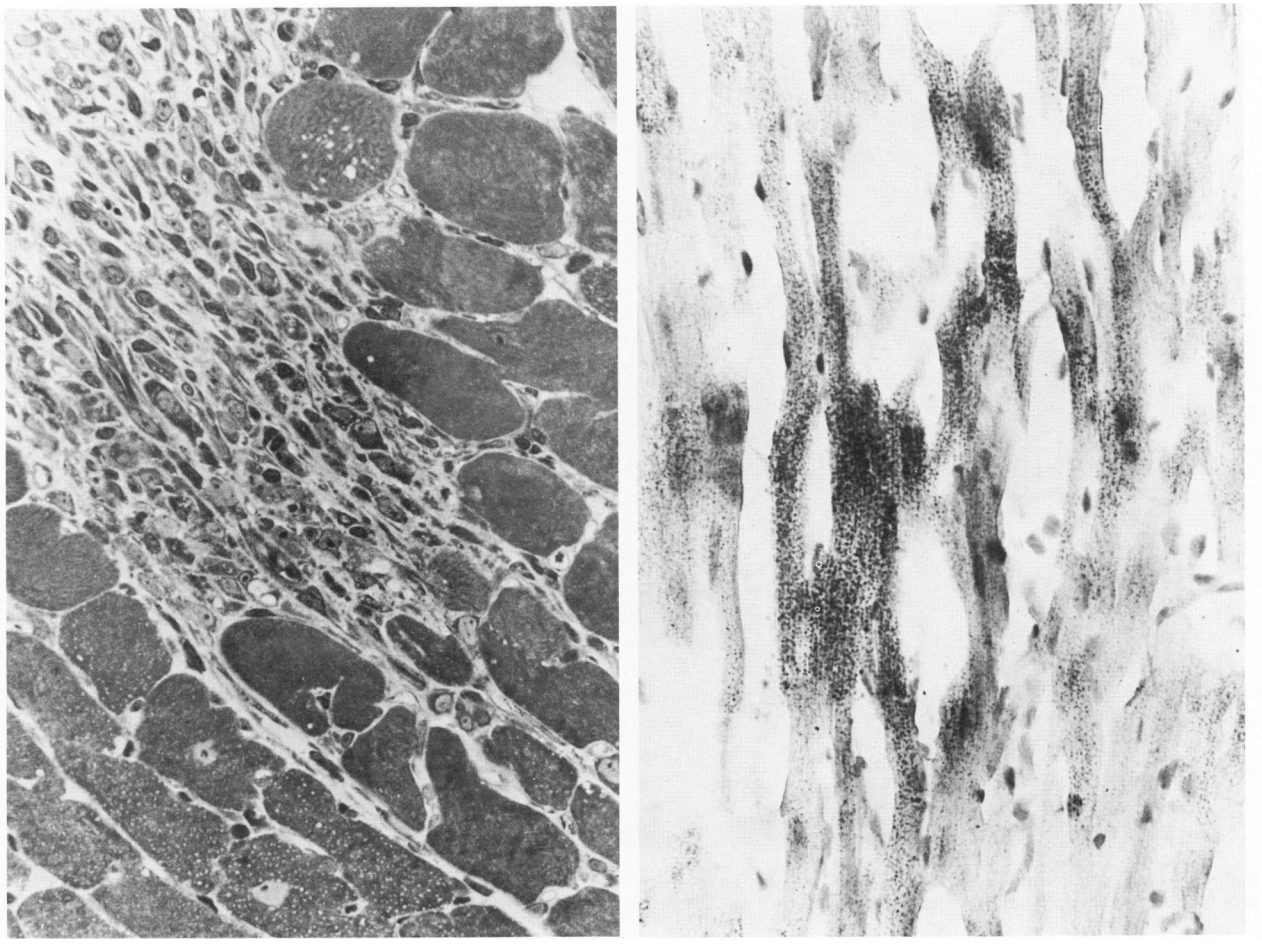

Figure 6—Light-microscopic photomicrographs of myocardium from rats fed a erucic acid diet for 72 days. **A**—There is a focus devoid of myocytes and infiltrated with macrophages. Adjacent myocytes are vacuolated. (Plastic section. Lee's stain, ×360) **B**—Several myocytes contain numerous lipid droplets. (Frozen section, oil red O, ×360)

erucyl CoA.[30] The present study supports these *in vitro* observations, in that there was a greater than 60% increase in long-chain fatty acyl CoA after *in vivo* erucic acid administration. However, studies by Dow-Walsh et al have demonstrated that mitochondria isolated from erucic-acid–treated myocardium have a normal *in vitro* oxidative capacity and adenosine triphosphate (ATP) production.[51]

Interestingly, this model of a diet-induced cardiac lipidosis closely resembles the accumulation of neutral lipid that has been described in the border zone of acute canine myocardial infarcts. Ligation of a coronary artery in canine myocardium results in the development of "lipid-laden" cells in the periphery of the myocardial infarcts.[26,27] In this zone, there is a marked accumulation of exogenous fatty acids in the form of triglycerides.[26,27] On electron-microscopic examination, the lipid droplets appear to be adjacent to, and in close contact with, the mitochondria. The underlying mechanism of neutral lipid accumulation probably is secondary to decreased fatty acid oxidation by heart mitochondria. In this case, the increase in tissue long-chain fatty acyl CoA is not due to the specific properties of the exogenous fatty acids, but rather is the result of a primary deficiency of molecular oxygen.[8] However, the net effect on the overall metabolism of neutral lipids is quite similar.

The apparent accumulation of neutral lipids in the transition zone of reversible to irreversible injury suggests that the accumulation of these lipids might be a direct mediator of irreversible injury and might be responsible for the marked accumulation of tissue Ca^{2+} that can accompany irreversible injury when a source of interstitial fluid is provided.[44,47,52] In addition, previous investigations of ischemia *in vivo* have demonstrated that the accumulation of long-chain acyl CoA closely correlated with an impairment of isolated mitochondrial respiration.[4,6,53] Similarly,

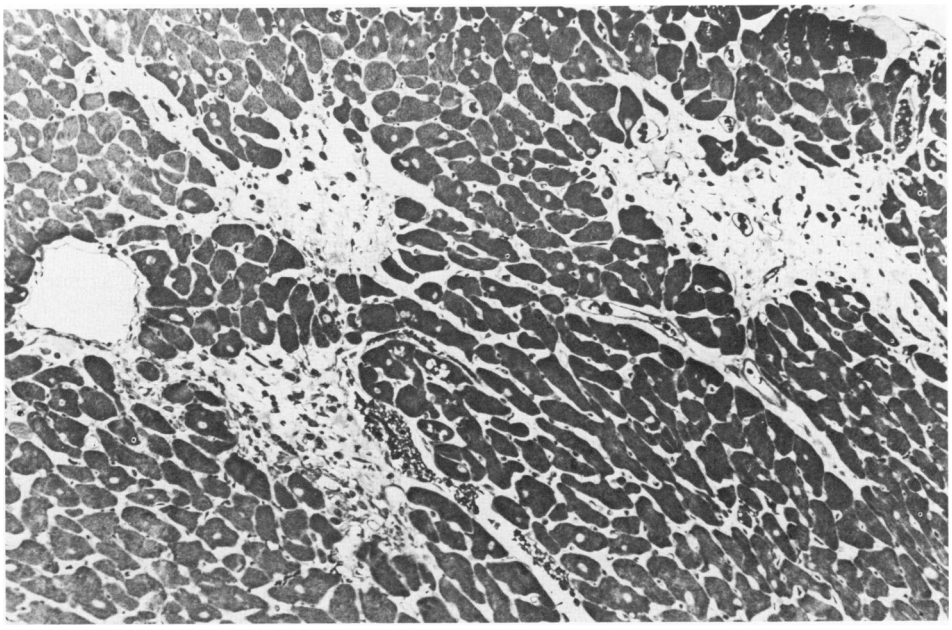

Figure 7 — Light-microscopic photomicrograph of myocardium from rat fed a erucic acid diet for 98 days. Focal small lesions are devoid of myocytes and are composed of loose fibrous tissue. (Plastic section, Lee's stain, ×144)

treatment of isolated mitochondria with exogenous long-chain acyl CoA resulted in a decrease in the mitochondrial respiratory-control index.[8]

However, the results of the present study suggest that acute increases in neutral lipids within the myocardial cell are indeed reversible. Acute administration of erucic acid produces a marked relative increase in neutral lipid content but does not result in histologic evidence of necrosis or of an increased tissue Ca^{2+} content. Thus, the accumulation of triglyceride and long-chain acyl CoA may be related more to the efficiency of fatty acid oxidation *per se*, than to the development of severe structural alterations and the potential for marked accumulation of tissue calcium that characterizes irreversible myocardial cell injury.[44,47,52] Because it has previously been demonstrated that myocardial sarcolemmal Ca^{2+} permeability is dependent on the maintenance of a normal phospholipid content,[2] it is likely that changes in the polar lipids during ischemia may be more important determinants of cellular Ca^{2+} homeostasis than the increases in neutral lipid content. This conclusion is supported by previous studies conducted on the mechanisms of toxic liver cell injury. The accumulation of large amounts of neutral lipids following the acute administration of alcohol[54] and ethionine[55] has been conclusively demonstrated to be reversible. On the other hand, the onset of accelerated phospholipid degradation in ischemic liver is associated with abnormal Ca^{2+} accumulation and irreversible hepatocellular damage.[1]

As has been found in previous studies,[28,29] the present study demonstrates that prolonged administration of erucic acid was associated with the development of multiple microscopic foci of myocardial necrosis, leading to myocyte loss and fibrosis. The mechanisms responsible for this chronic effect of erucic acid feeding require further study.

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